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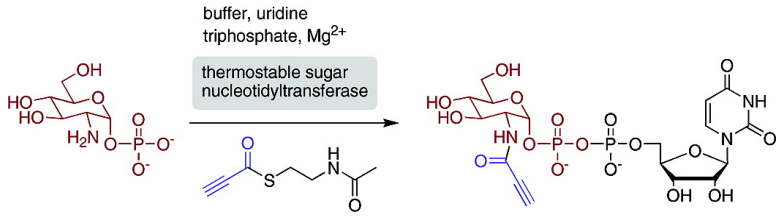

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One-Step Synthesis of Labeled Sugar Nucleotides for Protein *O*-GlcNAc Modification Studies by Chemical Function Analysis of an Archaeal Protein

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Cells use uridinediphospho-*N*-acetylglucosamine (UDP-GlcNAc) to transfer *N*-acetylglucosamine to an incredible range of proteins, including enzymes and transcription factors, as a transient post-translational modification implicated in signaling pathways.[1] However, the low concentration of this modification makes identification of glycosylated sites on proteins challenging.[1,2] Facile access to labeled sugar nucleotides would allow the modification to be tagged for easy isolation and mass spectrometry-based sequencing. Herein we present the chemical function analysis of a recombinant sugar nucleotidyltransferase from the hyperthermophile *Pyrococcus furiosus* and its use in the one-pot synthesis of chloroacetyl- and alkyne-tagged analogues of UDP-GlcNAc.

Sugar nucleotides for natural products and glycobiology studies have attracted much recent interest, but chemical strategies for their synthesis remain cumbersome.[1a,b,3] Synthetic strategies incorporating biocatalysts are often limited by the stability of the enzymes and their limited tolerance of nonnatural substrates.[3] The majority of these studies have focused on bacterial and eukaryotic proteins. The third branch of life, archaea,[4] provides a largely untapped genetic source of potential synthetic enzymes, many of which are predicted to be unusually thermostable.[5] The thermal vent archaea *P. furiosus* is of particular interest as a large amount of information is expected from a structural proteomics effort and will require correlation to verified chemical function data.[6] To explore the value of these proteins as glycobiology reagents, we copied a 1260 base pair *P. furiosus* gene annotated as a glucose-1-phosphate thymidylyltransferase using the polymerase chain reaction and ligated it into a vector that provides a polyhistidine tag at the C-terminus of the expressed protein for ease of purification by affinity chromatography.

To evaluate the function and substrate specificity of this new gene product, the enzyme was first tested with glucose-1-phosphate (**1**) and deoxythymidinetriphosphate (dTTP) using our recently developed mass-spectrometry (MS)-based assay.[7] Indeed, deoxythymidinephosphoglucose was formed as expected from the genome annotation (Figure 1). The optimal activity was at 80 °C and a pH of 7.5, reaction parameters which would inactivate any *E. coli* proteins that might have coeluted with the *P. furiosus* protein. A divalent cation was necessary for activity with Mg^{2+} serving best. Nonlinear regression analysis of reactions run in triplicate resulted in a $K_M = 8.1 \pm 0.7$ μM and $k_{cat} = 2.9$ (s^{-1}) ($k_{cat}/K_M = 0.35$ (μM^{-1} s^{-1}) using glucose-1-phosphate as a substrate (Table 1). The enzyme also showed substantial activity with uridinetriphosphate (UTP) as the nucleotide triphosphate.

The utility of this novel nucleotidyltransferase in the synthesis of sugar nucleotides was then tested by evaluating the transfer efficiency of six different sugar-1-phosphates. Surprisingly, the enzyme turned over a variety of substrates, including mannose-, glucosamine- (**3**), and *N*-acetylglucosamine-1-phosphate (**2**). Clearly, only an analysis of the kinetic competence of these substrates would

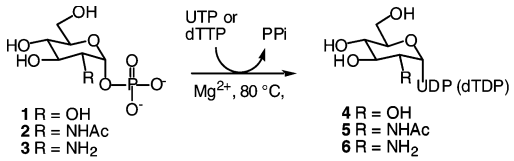

Figure 1. Synthesis of sugar nucleotides by an enzyme from *P. furiosus*.

Table 1. Kinetic Values for Each of the Functions of the *P. furiosus* Protein Assayed Independently with Varying Concentrations of One Substrate (Fixed Substrate Concentration in Parentheses)

substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (μM^{-1} s^{-1})
Nucleotidyltransferase			
glucose-1-phosphate (UTP)	9 ± 1	0.5	0.05
glucose-1-phosphate (dTTP)	8 ± 1	2.9	0.35
mannose-1-phosphate (UTP)	40 ± 4	1.2	0.03
mannose-1-phosphate (dTTP)	44 ± 5	1.4	0.03
N-acetylglucosamine-1-phosphate (UTP)	100 ± 10	44	0.45
N-acetylglucosamine-1-phosphate (dTTP)	90 ± 10	40	0.42
Acetyltransferase			
glucosamine-1-phosphate (**7**)	63 ± 9	9.5	0.15
7 (glucosamine-1-phosphate)	74 ± 7	6.5	0.08
8 (glucosamine-1-phosphate)	44 ± 4	1.5	0.03

delineate the most likely in vivo chemical function of this gene product (Table 1). The k_{cat}/K_M values calculated from three independent experiments indicated that *N*-acetylglucosamine-1-phosphate was actually a better substrate than glucose or mannose in the synthesis of both UDP- and dTDP-activated sugars.

The high kinetic competence of this enzyme with *N*-acetylglucosamine-1-phosphate hinted that this enzyme might actually be a bifunctional enzyme.[8] These enzymes catalyze both the acylation of glucosamine-1-phosphate (Figure 2) and the subsequent nucleotide transfer. The standard assay for this acyltransferase activity is the indirect detection of released thiols using Ellman's reagent.[9] Because this assay measures the decomposition of thioesters by a variety of pathways that do not lead to product, we reasoned that an assay that directly measured product formation would be preferable, especially at elevated temperatures. Reaction analysis by ESI-MS rapidly confirmed our hypothesis. In the presence of acetyl coenzyme A but in the absence of UTP, peaks corresponding to glucosamine-1-phosphate ($m/z = 258$) disappeared over time as a peak corresponding to *N*-acetylglucosamine-1-phosphate ($m/z = 300$) appeared with the enzyme present. Acylation precedes nucleotide addition. No change was observed in the absence of the enzyme. With the addition of UTP, too, glucosamine-1-phosphate was converted to UDP-GlcNAc. Peak quantification with internal standards and calibration curves provides the first MS-based acyltransferase assay. To probe the steric limitations of the acyl moiety, propyl CoA and butyryl CoA were tested. As was found

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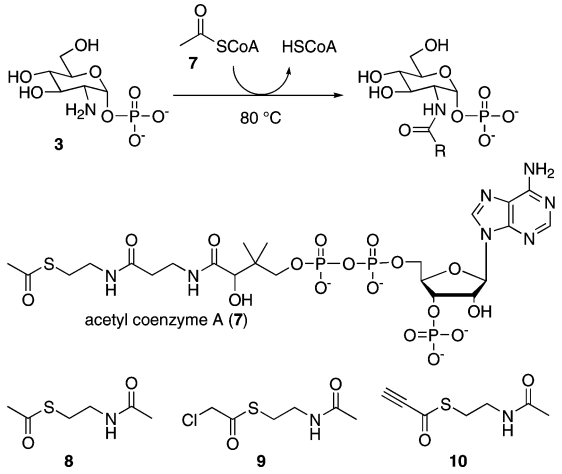

Figure 2. Acyltransferase activity of the bifunctional enzyme with natural and truncated acetylCoA derivatives. R = CH_3, CH_2Cl, CCH.

in the analogous bacterial enzyme,[10] the enzyme tolerated a propyl group reasonably well with only an 8-fold decrease in k_{cat}/K_M, but a two-carbon extension proved detrimental to efficient product formation.

Reexamination of the protein sequence indeed shows alignment of the first 240 amino acids with NTP-transferases. However, the C-terminal portion contains a hexapeptide repeat motif often associated with acyltransferase activity.[11] Analysis of a structure of the analogous bifunctional enzyme from *Escherichia coli* with bound acetyl CoA (**7**) shows several contacts between CoA and the protein. However, no contacts are made with the acyl side chain, and the diphosphate is solvent exposed.[12] Fatty acid synthases (FASs) and polyketide synthases (PKSs) are known to accept truncated CoA thioesters as substrates, but these enzymes form covalent acylated intermediates before transfer to a bound substrate.[13] The structure of the bifunctional *E. coli* enzyme does not support a covalent enzyme intermediate for the acyltransfer reaction to glucosamine.

The ability of FASs and PKSs to accept significantly cheaper and less synthetically challenging analogues such as the *N*-acetylcysteamine thioester **8** has greatly aided experiments to understand and exploit these enzymes; therefore, we decided to synthesize and test **8** with the *P. furiosus* enzyme. In fact, this truncated analogue was readily accepted and turned over with only a 2−3-fold reduction in k_{cat}/K_M compared to the full length substrate (Table 1). Emboldened by this discovery, we synthesized analogues **9** and **10** (Figure 2) containing sterically conservative modifications that are nevertheless amenable to selective tagging reactions via thiol substitution[14] or Sonogashira reactions[15] and alkyne/azide coupling,[16] respectively. The enzyme not only was able to acylate the amine but also could convert the resulting modified glucosamine-1-phosphate into the activated UDP-sugar with 80% conversion in one pot. The acylation reactions did not occur at 37 °C with enzyme or at 80 °C without enzyme with one exception. Significant uncatalyzed acylation took place with the alkyne analogue at 80 °C. Therefore, even if the acyltransfer domain did not readily accept this modification, the thermostability of the enzyme allows the coupling of the chemical acylation with the enzymatic uridyl transfer in one pot. For comparison, the chemical synthesis of an azide-labeled UDP-GlcNAc required 11 chemical steps with less than a 15% overall yield.[1b]

Archaeal genomes clearly are a rich source of proteins; however, genome annotation based on primary sequence homologies sometimes does not even hint at the true synthetic potential of the corresponding proteins. Chemical function analysis is required, ideally, with a range of substrates to ascertain the most likely in vivo function and to uncover unexpected properties. The archaeal gene denoted originally as a glucose-1-phosphate deoxythymidylyltransferase is actually a bifunctional acetyltransferase/*N*-acetylglucosamine-1-phosphate uridylyltransferase. The use of thermostable enzymes in synthetic schemes also allows chemical steps that are sluggish at lower temperatures to be incorporated into the same step with an enzyme. Current studies are underway to use these tagged sugar nucleotides to probe protein modifications by *N*-acetylglucosamine moieties.

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Supporting Information Available: Experimental details for cloning, expression, and purification of the hyperthermophilic enzyme, details of the mass spectrometry assays including calibration curves, and synthetic details for thioesters **9** and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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